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DOI: 10.14670/HH-18-398
Article type: ORIGINAL ARTICLE
Accepted: 2021-11-26
Epub ahead of print: 2021-11-26

This article has been peer reviewed and published immediately upon acceptance.
Articles in “Histology and Histopathology” are listed in Pubmed.
Pre-print author’s version
Circ-SFMBT2 facilitates the malignant growth of acute myeloid leukemia cells by modulating miR-582-3p/ZBTB20 pathway

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Running title: The role of circ-SFMBT2 in acute myeloid leukemia
Abstract

Background: Acute myeloid leukemia (AML) is a highly heterogeneous hematological malignancy. Circular RNAs (circRNAs) play crucial roles in AML progression. This study aimed to explore the function and potential mechanism of circRNA Sem like with four mbt domains 2 (circ-SFMBT2; hsa_circ_0017639) in AML.

Methods: The levels of circ-SFMBT2, microRNA-582-3p (miR-582-3p) and zinc finger and BTB domain containing 20 (ZBTB20) were measured by quantitative real-time PCR and Western blot. Cell Counting Kit-8 (CCK-8), colony formation, flow cytometry and transwell assays were used to evaluate cell proliferation, apoptosis, migration and invasion. Glycolysis was assessed by detecting glucose consumption, lactate production and ATP/ADP ratios. The related protein expression was examined by Western blot. The binding relationship between miR-582-3p and circ-SFMBT2/ZBTB20 was verified by dual-luciferase reporter assay.

Results: Circ-SFMBT2 and ZBTB20 levels were elevated, while miR-582-3p level was reduced in AML patients and cells. Depletion of circ-SFMBT2/ZBTB20 impeded proliferation, migration, invasion and glycolysis and induced apoptosis in AML cells. Moreover, circ-SFMBT2 facilitated AML progression by sponging miR-582-3p, and miR-582-3p targeted ZBTB20 to hinder AML development.

Conclusion: Knockdown of circ-SFMBT2 suppressed AML progression by regulating the miR-582-3p/ZBTB20 axis, which might provide a potential therapeutic strategy for AML.

Keywords: Acute myeloid leukemia, circ-SFMBT2, miR-582-3p, ZBTB20
Introduction

Acute myeloid leukemia (AML) is a hematologic malignancy characterized by uncontrolled proliferation derived from hematopoietic stem/progenitor cells (Khwaja et al., 2016). Due to the complex heterogeneity of AML, the treatment of AML is challenging and the prognosis of AML patients is poor (Carter et al., 2020). Conventional treatments for AML patients include chemotherapy and hematopoietic stem cell transplantation (Short et al., 2018). With great advances in genomics and insight into the mechanisms of chemotherapeutic resistance, the treatment strategy of AML is being rapidly developed (Short et al., 2020). Hence, identifying new molecular therapeutic targets is critical to improving clinical outcomes in patients with AML.

Circular RNAs (circRNAs) are endogenous RNA molecules with unique closed-loop structures produced by back-splcing (Qian et al., 2018). Because of their widespread presence in eukaryotic cells, circRNAs have been identified as valuable biomarkers for cancer (Shang et al., 2019b). Recently, substantial literature has unveiled the important roles of circRNAs in hematological malignancies, including AML (Guo et al., 2020; Perez de Acha et al., 2020). For example, circ_0009910 increased the resistance of chronic myeloid leukemia cells to imatinib by triggering autophagy (Cao et al., 2020a). Wu et al. revealed that circ_0132266 decelerated cell growth in chronic lymphocytic leukemia through combining with microRNA-337-3p (Wu et al., 2019). In addition, aberrant expression of circRNAs is strongly related to AML progression (Jamal et al., 2019). In a recent report, high-throughput sequencing results showed that hsa_circ_0017639 (circ-SFMBT2) derived from the Scom like with four mbt domains 2 (SFMBT2) gene was strikingly up-regulated in AML (Yi et al., 2021). Nonetheless, the specific role and potential mechanism of circ-SFMBT2 in AML are still unclear.

Moreover, plentiful studies have identified that circRNAs overturn the inhibition of microRNAs (miRNAs) on their target genes by functioning as miRNA sponges (Zhong et al., 2018). Besides, miRNAs exert crucial regulatory effects on the occurrence and development of AML, and are effective targets for AML treatment (Marcucci et al.,
For instance, hsa-miR-12462 enhanced the sensitivity of AML cells to cytarabine by negatively modulating SLC9A1 (Jia et al., 2020). Also, miR-4792 hindered the growth and metastasis of AML cells and accelerated apoptosis through down-regulating Kindlin-3 (Qin et al., 2020). Additionally, miR-582-3p was reduced in AML patients and cells, and its overexpression restrained AML development by competitively binding to cyclin B2 (Li et al., 2019). However, the regulatory network of miR-582-3p in AML is still largely unknown.

In the current research, we elucidated the expression pattern of circ-SFMBT2 in AML. Furthermore, the exact function and possible downstream targets of circ-SFMBT2 were further explored in AML cells. These findings revealed a new circ-SFMBT2/miR-582-3p/zinc finger and BTB domain containing 20 (ZBTB20) regulatory pathway.

Materials and methods

Patients and samples

AML patients (n=27) and healthy volunteers (n=27) were recruited from Puren Hospital Affiliated to Wuhan University of Science and Technology. Serum samples were obtained from all AML patients and healthy controls. All subjects signed written informed consent, and this protocol was approved by the Ethics Committee of Puren Hospital Affiliated to Wuhan University of Science and Technology. AML patients receive no medical treatment prior to this research.

Cell culture

AML cell lines (U937, NB4, K562 and HL-60) and human bone marrow stromal cell line (HS-5) were commercially acquired from BeNa Culture Collection (Beijing, China). AML cell lines were incubated in RPMI-1640 medium (Hyclone, Logan, UT, USA) harboring 10% fetal bovine serum (FBS; Hyclone). Additionally, HS-5 cells
were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Hyclone) containing 10% FBS (Hyclone). All cells were cultured in an incubator with 5% CO₂ at 37°C.

**Cell transfection**

Small interfering RNA against circ-SFMBT2 or ZBTB20 (si-circ-SFMBT2 or si-ZBTB20) and the control (si-NC), miR-582-3p mimics (miR-582-3p) and negative control (miR-NC), circ-SFMBT2 overexpression vector (circ-SFMBT2) and the control (pCD5-ciR), miR-582-3p inhibitor (anti-miR-582-3p) and negative control (anti-miR-NC), ZBTB20 overexpression vector (ZBTB20) and empty vector (pcDNA) were purchased from Genechem (Shanghai, China). Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was applied for cell transfection when cell confluence was about 80%.

**Quantitative real-time PCR (qRT-PCR)**

First, TRIzol reagent (Leagene, Beijing, China) was utilized for RNA extraction. In addition, 2 µg of RNA was reacted with RNase R (3 U/µg; Seebio, Shanghai, China) in the RNase R digestion test. Meanwhile, the nucleus and cytoplasm of K562 and HL-60 cells were separated with PARIS kit (Invitrogen). Afterwards, cDNA was obtained with the certain cDNA synthesis kit (Vazyme, Nanjing, China). Then, qRT-PCR was implemented via SYBR Green Master Mix (Vazyme). GAPDH and U6 (for miR-582-3p) were used as endogenous controls. The primers are displayed below:

- circ-SFMBT2-F: 5’-CGTCGGTGACTAAGCAATCA-3’, circ-SFMBT2-R: 5’-CTATAGGGCCTTTCCCTCGC-3’;
- SFMBT2-F: 5’-TCTGCGCTACTGCGGTTAC-3’, SFMBT2-R: 5’-ACCAGTCAAGTCACGTATGAGAA-3’;
- miR-582-3p-F: 5’-GCTGGGTAACTGGTTGAACAAC-3’, miR-582-3p-R: 5’-TGGTGTCGTGGAGTCG-3’;
- ZBTB20-F: 5’-GCGACCACCACATGGAAGA-3’, ZBTB20-R: 5’-TTGCCCACTAGGGTCTGGA-3’;
- GAPDH-F: 5’-GCACCGTCAAGGCTGAGAAC-3’, GAPDH-R: 5’-
ATGGTGGTGTTAAGACGCCAGT-3'; U6-F: 5'-CTCGCTTCGCCAGCACATA-3',
U6-R: 5'-AACGCTTCACGAATTTGCGT-3'.

**CCK-8 assay**

K562 and HL-60 cells (3000 cells/well) were seeded into 96-well plates and cultured for 48 h. Then, 10 µL of Cell Counting Kit-8 (CCK-8; Boster, Wuhan, China) was added to each well and incubated for 4 h. Subsequently, cell viability was detected by measuring the optical density at 450 nm via Microplate Reader (Bio-Rad, Hercules, CA, USA).

**Colonie formation assay**

After transfection, K562 and HL-60 cells (3000 cells/well) were seeded into 96-well plates and cultured for 14 days. The colonies were fixed with formaldehyde (Solarbio, Beijing, China) and stained with 0.1% crystal violet (Solarbio). Finally, the colonies were counted under a microscope (Olympus, Tokyo, Japan).

**Flow cytometry**

Cell apoptosis was monitored utilizing Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China). Briefly, transfected K562 and HL-60 cells were harvested and washed with phosphate buffer saline (PBS; Solarbio). Following staining with Annexin V-FITC and Propidium Iodide (PI), the apoptotic cells were detected via flow cytometer (Beckman Coulter, Miami, FL, USA).

**Transwell assay**

Cell invasion and migration abilities were determined by transwell inserts (Corning, Corning, NY, USA) equipped with or without Matrigel (Corning). In brief, AML cells suspended in serum-free medium were placed in the upper chamber. Meanwhile, the bottom chamber was filled with AML cells suspended in the medium containing 10%...
FBS (Hyclone). After 24 h of cultivation, the transferred cells were stained with 0.1% crystal violet (Solarbio) and counted under a microscope (Olympus) at 100× magnification.

**Western blot assay**
After extracting with RIPA lysis buffer (Beyotime), the protein was quantified using BCA Protein Assay Kit (Beyotime). Subsequently, the protein was separated by 10% SDS-PAGE and transferred onto PVDF membranes (Beyotime). After blocking with 5% skimmed milk for 2 h, the membranes interacted with primary antibodies against CyclinD1 (1:3000, ab226977, Abcam, Cambridge, UK), Bcl-2 associated X protein (Bax; 1:1000, ab104156, Abcam), matrix metalloproteinase 9 (MMP9; 1:1000, ab38898, Abcam), ZBTB20 (1:2000, ab127702, Abcam) or GAPDH (1:2500, ab9485, Abcam). Afterwards, the membranes were probed with a secondary antibody (1:25000, ab205718, Abcam). Lastly, the protein bands were tested using ECL reagent (Absin, Shanghai, China).

**Detection of glucose consumption, lactate production and ATP/ADP ratios**
Relative glucose consumption and lactate production were determined in K562 and HL-60 cell culture medium by using Glucose Assay Kit and Lactic Acid Kit (Abcam) following the manufacturer’s requirements. Relative ATP/ADP ratios were detected by using ApoSENSOR ADP/ATP Ratio Assay Kit (BioVision, Palo Alto, CA, USA) according to the supplier’s protocol.

**Dual-luciferase reporter assay**
The sequences of circ-SFMBT2 or ZBTB20 3’UTR harboring the putative miR-582-3p binding site were inserted into pmirGLO vector (LMAI Bio, Shanghai, China), termed as WT-circ-SFMBT2 and WT-ZBTB20 3’UTR. Besides, circ-SFMBT2 or ZBTB20 3’UTR sequences harboring the mutant binding site were inserted into
pmirGLO vector (LMAI Bio) to form MUT-circ-SFMBT2 and MUT-ZBTB20 3’UTR. Afterwards, the constructed plasmids and miR-582-3p or miR-NC were introduced into AML cells. The luciferase activity was tested using Dual-Lucy Assay Kit (Solarbio).

**Statistical analysis**

All data are shown as mean ± standard deviation in three independent replicates using GraphPad Prism 7 software (GraphPad, San Diego, CA, USA). The differences were determined by Student’s t-test and one-way analysis of variance. The linear relationship between miR-582-3p and circ-SFMBT2/ZBTB20 was confirmed by Spearman’s correlation analysis. $P<0.05$ was considered statistically significant.

**Results**

**Circ-SFMBT2 was up-regulated in AML**

To investigate the potential function of circ-SFMBT2 in AML, we first detected the expression of circ-SFMBT2 in AML patients and cell lines. According to GSE94591 expression dataset, hsa_circRNA_100542 (circ-SFMBT2; hsa_circ_0017639) was strikingly up-regulated in AML patients compared with normal controls (Figure 1A).

In addition, circ-SFMBT2 expression was determined in serum from AML patients (n=27) and healthy controls (n=27). As shown in Figure 1B, circ-SFMBT2 level was remarkably increased in AML patients relative to healthy volunteers. Simultaneously, circ-SFMBT2 level in AML cell lines (U937, NB4, K562 and HL-60) was prominently elevated in comparison to human bone marrow stromal cell line HS-5 (Figure 1C).

RNase R digestion assay suggested that circ-SFMBT2 was more resistant to RNase R than linear SFMBT2 (Figure 1D and 1E). In addition, nuclear and cytoplasmic fraction assay revealed that circ-SFMBT2 was mainly distributed in the cytoplasm (Figure 1F and 1G). These data demonstrated that circ-SFMBT2 might play a carcinogenic role in AML.
Depletion of circ-SFMBT2 inhibited the proliferation, migration, invasion and glycolysis of AML cells and induced apoptosis

To explore the biological function of circ-SFMBT2 in AML cells, loss-of-function experiments were implemented in K562 and HL-60 cells by silencing circ-SFMBT2. Compared with the si-NC group, si-circ-SFMBT2 transfection significantly reduced the expression of circ-SFMBT2 in AML cells (Figure 2A). CCK-8 assay showed that down-regulation of circ-SFMBT2 inhibited the viability of K562 and HL-60 cells (Figure 2B). Simultaneously, colony formation assay suggested that the introduction of si-circ-SFMBT2 markedly reduced the number of colonies compared with the si-NC group (Figure 2C). Additionally, interference of circ-SFMBT2 remarkably increased the apoptosis rate of K562 and HL-60 cells (Figure 2D). Transwell assay revealed that circ-SFMBT2 silencing significantly reduced the migration and invasion capabilities of AML cells compared to the control group (Figure 2E and 2F). Besides, transfection of si-circ-SFMBT2 remarkably reduced CyclinD1 and MMP9 levels and prominently elevated Bax level, suggesting that circ-SFMBT2 silence suppressed the proliferation and metastasis of AML cells and triggered apoptosis (Figure 2G and 2H). Additionally, circ-SFMBT2 depletion attenuated glycolysis by reducing glucose consumption, lactate production and ATP/ADP ratios (Figure 2I-2K). These data evidenced that knockdown of circ-SFMBT2 hindered the malignant phenotypes of AML cells.

Circ-SFMBT2 directly interacted with miR-582-3p

As depicted in Figure 3A, the possibility of circ-SFMBT2 binding to miR-582-3p was predicted using the circinteractome database (https://circinteractome.irp.nia.nih.gov/). Firstly, qRT-PCR analysis showed that miR-582-3p overexpression efficiency was significant (Figure 3B). Next, the binding relationship was validated by dual-luciferase reporter assay, and the results revealed that miR-582-3p mimics markedly reduced the luciferase activity of WT-circ-SFMBT2 reporter in K562 and HL-60 cells (Figure 3C and 3D). In addition, miR-582-3p exhibited a lower expression in serum from AML...
patients than healthy controls (Figure 3E). Spearman’s correlation analysis showed a negative correlation between circ-SFMBT2 and miR-582-3p levels in serum from AML patients (Figure 3F). Also, miR-582-3p expression was strikingly reduced in K562 and HL-60 cells compared with HS-5 cells (Figure 3G). As shown in Figure 3H, transfection with circ-SFMBT2 resulted in a significant increase in circ-SFMBT2 level compared to the pCD5-ciR group. Moreover, interference of circ-SFMBT2 promoted the expression of miR-582-3p, while up-regulation of circ-SFMBT2 restrained the expression of miR-582-3p (Figure 3I). Thus, these results indicated that circ-SFMBT2 negatively regulated miR-582-3p.

Knockdown of miR-582-3p overturned the effect of circ-SFMBT2 silence on AML cell progression

To clarify whether circ-SFMBT2 sponged miR-582-3p to regulate AML development, rescue experiments were carried out in AML cells transfected with si-circ-SFMBT2 or/and anti-miR-582-3p. Firstly, the level of miR-582-3p was markedly reduced after transfection with anti-miR-582-3p in AML cells (Figure 4A). As displayed in Figure 4B, the introduction of anti-miR-582-3p overturned the increase in miR-582-3p level caused by circ-SFMBT2 depletion. In addition, CCK-8 and colony formation assays showed that co-transfection of si-circ-SFMBT2 and anti-miR-582-3p partially restored the repressive effect of circ-SFMBT2 knockdown on AML cell proliferation (Figure 4C and 4D). Flow cytometry and transwell assays exhibited that circ-SFMBT2 silencing induced apoptosis and suppressed migration and invasion in K562 and HL-60 cells, while these impacts were reversed by inhibiting miR-582-3p (Figure 4E-4G). Furthermore, the levels of CyclinD1, Bax and MMP9 were detected in AML cells to confirm the effects of circ-SFMBT2/miR-582-3p axis on AML cell progression. The results suggested that interference of circ-SFMBT2 led to a marked decrease in CyclinD1 and MMP9 levels and a significant increase in Bax level, whereas addition of anti-miR-582-3p partially abolished these changes (Figure 4H and 4I). In addition,
miR-582-3p knockdown restored the inhibition of circ-SFMBT2 silence on glycolysis by increasing glucose consumption, lactate production and ATP/ADP ratios (Figure 4J-4L). These data evidenced that silence of circ-SFMBT2 impeded AML cell progression by modulating miR-582-3p.

**MiR-582-3p directly targeted ZBTB20**

Next, the TargetScan database (http://www.targetscan.org/vert_72/) predicted that miR-582-3p and ZBTB20 3’UTR had a possible binding site (Figure 5A). Subsequently, dual-luciferase reporter assay suggested that miR-582-3p overexpression strikingly reduced the luciferase activity of WT-ZBTB20 3’UTR reporter in AML cells (Figure 5B and 5C). Additionally, we found that ZBTB20 mRNA level was remarkably elevated in serum from AML patients (n=27) relative to healthy controls (Figure 5D). As illustrated in Figure 5E, previous data indicated that ZBTB20 was highly expressed in AML patients compared with normal controls. Spearman’s correlation coefficient showed that miR-582-3p was negatively correlated with ZBTB20 mRNA in serum from AML patients (Figure 5F). In addition, ZBTB20 protein level in K562 and HL-60 cells was significantly higher than that in HS-5 cells (Figure 5G). Moreover, up-regulation of miR-582-3p inhibited the protein expression of ZBTB20, while knockdown of miR-582-3p had the opposite effect (Figure 5H). These data indicated that miR-582-3p directly interacted with ZBTB20.

**Silencing of ZBTB20 suppressed the malignant phenotypes of AML cells**

We also explored the role of ZBTB20 in AML cells by interfering ZBTB20. As shown in Figure 6A, ZBTB20 protein level in the si-ZBTB20 group was prominently decreased compared to the si-NC group. A series of loss-of-function experiments revealed that silencing of ZBTB20 inhibited cell viability (Figure 6B), colony formation (Figure 6C), cell migration and invasion (Figure 6E-6F), and accelerated cell apoptosis (Figure 6D) in K562 and HL-60 cells. Meanwhile, Western blot analysis
suggested that CyclinD1 and MMP9 levels were markedly reduced, and Bax level was remarkably increased in the si-ZBTB20 group compared with the control group (Figure 6G-6H). Furthermore, interference of ZBTB20 suppressed the glycolysis of AML cells (Figure 6I-6J). Overall, these data evidenced that depletion of ZBTB20 hindered the progression of AML cells.

**ZBTB20 abolished the effect of miR-582-3p on AML cell progression**

To elucidate whether miR-582-3p targeted ZBTB20 to influence AML development, K562 and HL-60 cells were introduced with miR-582-3p mimics or/and ZBTB20 to perform rescue experiments. As displayed in Figure 7A, ZBTB20 overexpression efficiency was determined by Western blot assay. In addition, co-transfection of miR-582-3p and ZBTB20 restored the reduction in ZBTB20 protein level caused by miR-582-3p mimics (Figure 7B). CCK-8 and colony formation assays exhibited that introduction of ZBTB20 rescued the suppressive effect of miR-582-3p mimics on AML cell proliferation (Figure 7C-7D). Moreover, miR-582-3p overexpression promoted apoptosis and restrained migration and invasion in AML cells, whereas co-transfection of miR-582-3p and ZBTB20 alleviated these effects (Figure 7E-7G). Consistently, ZBTB20 transfection partially abrogated the decrease in CyclinD1 and MMP9 levels and the increase in Bax level induced by miR-582-3p up-regulation (Figure 7H-7I). Besides, up-regulation of ZBTB20 recovered the suppressive effect of miR-582-3p overexpression on glycolysis in AML cells (Figure 7J-7L). These data demonstrated that miR-582-3p inhibited AML cell proliferation through targeting ZBTB20.

**Circ-SFMBT2 regulated ZBTB20 expression by sponging miR-582-3p**

To investigate the interaction between ZBTB20 and the circ-SFMBT2/miR-582-3p axis, ZBTB20 protein level was examined in K562 and HL-60 cells transfected with si-circ-SFMBT2 or/and anti-miR-582-3p. Western blot analysis showed that circ-SFMBT2 knockdown significantly decreased ZBTB20 protein level, which was restored by
down-regulating miR-582-3p (Figure 8A and 8B). These data indicated that circ-SFMBT2 regulated ZBTB20 expression by absorbing miR-582-3p.

**Discussion**

Due to the heterogeneity and complex pathogenesis of AML, the exact mechanism of AML development has not been fully elucidated. Therefore, exploring the potential mechanism of AML progression is very important for determining the diagnostic and prognostic targets of AML. Recently, plentiful studies have corroborated the critical role of circRNA dysregulation in AML progression (Liu et al., 2019). For example, circPAN3 expedited AML drug resistance by mediating autophagy through AMPK/mTOR signaling (Shang et al., 2019a). Likewise, circ_100290 accelerated the growth of AML cells via regulating the miR-203/Rab10 pathway (Fan et al., 2018). Additionally, circ-SFMBT2 contributed to the malignant growth of gastric carcinoma by absorbing miR-182-5p and activating CREB1 (Sun et al., 2018). In the present research, we disclosed that circ-SFMBT2 expression was prominently elevated in AML patients and cells. Furthermore, silence of circ-SFMBT2 restrained the progression of AML cells.

Moreover, accumulating evidence has identified that circRNAs participate in the occurrence and development of tumors by affecting miRNA sponge activity (Cao et al., 2020b; Tang and Hann, 2020). In acute myeloid leukemia, several circRNAs regulate tumor progression by acting as sponges for miRNAs. For example, circRNF13 hindered the growth and metastasis of AML cells by repressing miRNA-1224-5p (Zhang et al., 2020b). Yi et al. found that circ-PTK2 triggered the malignant phenotypes of AML by sponging miR-330-5p to elevate FOXM1 expression (Yi et al., 2021). In addition, we selected miR-582-3p as the molecular target of circ-SFMBT2 based on previous research. Furthermore, some studies have verified that miR-582-3p is a suppressing-factor in many cancers. For instance, up-regulation of miR-582-3p restrained bone metastasis in prostate carcinoma by mediating TGF-β signaling (Huang et al., 2019).
Also, circ_HIPK3 accelerated the progression of hepatocellular carcinoma through competitively regulating miR-582-3p (Zhang et al., 2020a). In the current research, we unveiled that circ-SFMBT2 inversely modulated miR-582-3p. Moreover, rescue experiments indicated that circ-SFMBT2 facilitated AML progression by absorbing miR-582-3p.

Additionally, mounting evidence has validated that miRNAs bind to mRNAs to inhibit their translation and activity, thereby playing a key role in various diseases (Di Leva et al., 2014; Treiber et al., 2019). In this research, we disclosed that ZBTB20 directly targeted miR-582-3p through bioinformatics analysis and experimental verification. ZBTB20 belongs to the POK (POZ and Krüppel) family of transcriptional repressors (Sutherland et al., 2009). In glioblastoma, ZBTB20 expression was strikingly elevated, and miR-758-5p overexpression impeded tumor progression by combining with ZBTB20 (Liu et al., 2018). In gastric cancer, ZBTB20 aggravated the malignant properties of tumor cells through activating the NF-κB pathway (Zhang et al., 2019). Besides, a previous report revealed that LINC00641 contributed to AML cell growth and migration via sponging miR-378a and increasing ZBTB20 expression (Wang et al., 2019). In our research, ZBTB20 expression was conspicuously up-regulated in AML patients and cells. Importantly, miR-582-3p blocked AML development by modulating ZBTB20.

In conclusion, we unveiled that circ-SFMBT2 expedited the growth and metastasis of AML cells and restrained cell apoptosis. In terms of mechanism, circ-SFMBT2 facilitated AML progression by absorbing miR-582-3p to up-regulate ZBTB20. These findings might provide a promising therapeutic target for AML.

Disclosure of interest

The authors declare that they have no financial conflicts of interest.
Acknowledgement

None.

Funding

This work was supported by Natural Science Foundation of China (No. 81873444) and Natural Science Foundation of China (No. 82070213) and Key R&D Projects of Hubei Province (No.2020BCB021).

Availability of data and materials

Please contact the correspondence author for data request and raw data.

Ethics approval and consent participate

Written informed consent was obtained from patients with approval by the Institutional Review Board in Puren Hospital Affiliated to Wuhan University of Science and Technology.

References


Figure legends

Figure 1. Expression of circ-SFMBT2 in AML. (A) The heat map displayed abnormally expressed circRNAs in healthy controls (n=4) and AML patients (n=6). (B) The expression of circ-SFMBT2 in serum from AML patients (n=27) and healthy controls (n=27) was measured by qRT-PCR. (C) The level of circ-SFMBT2 was detected in human bone marrow stromal cell line HS-5 and AML cell lines (U937, NB4, K562 and HL-60) via qRT-PCR. (D and E) After RNase R treatment, the levels of circ-SFMBT2 and linear SFMBT2 in K562 and HL-60 cells were examined by qRT-PCR. (F and G) The abundance of circ-SFMBT2 in the nucleus and cytoplasm was determined using qRT-PCR. *P < 0.05, ***P < 0.001, ****P < 0.0001.

Figure 2. Effect of circ-SFMBT2 knockdown on AML cells. K562 and HL-60 cells were transfected with si-NC or si-circ-SFMBT2. (A) The expression of circ-SFMBT2 was tested by qRT-PCR. (B) Cell viability was detected using CCK-8 method. (C) Cell proliferation ability was assessed by colony formation assay. (D) The apoptosis rate of K562 and HL-60 cells was evaluated by flow cytometry. (E and F) Transwell assay was utilized to detect cell migration and invasion. (G and H) The protein levels of CyclinD1, Bax and MMP9 were measured using Western blot assay. (I-K) Relative glucose consumption, lactate production and ATP/ADP ratios were detected using the appropriate kit. **P < 0.01, ***P < 0.001, ****P < 0.0001.
Figure 3. Circ-SFMBT2 directly interacted with miR-582-3p. (A) The putative binding site between circ-SFMBT2 and miR-582-3p was shown by circinteractome. (B) The transfection efficiency of miR-582-3p mimics was detected using qRT-PCR. (C and D) The binding relationship was verified by dual-luciferase reporter assay. (E) The level of miR-582-3p in serum from AML patients (n=27) and healthy volunteers (n=27) was examined using qRT-PCR. (F) The correlation between circ-SFMBT2 and miR-582-3p was tested by Spearman’s correlation analysis. (G) miR-582-3p level in HS-5 cells and AML cells (K562 and HL-60) was measured by qRT-PCR. (H) The overexpression efficiency of circ-SFMBT2 was detected by qRT-PCR. (I) miR-582-3p level was examined by qRT-PCR in K562 and HL-60 cells transfected with si-NC, si-circ-SFMBT2, pCD5-ciR or circ-SFMBT2. **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 4. Knockdown of miR-582-3p overturned the effect of circ-SFMBT2 silence on AML cell progression. (A) The transfection efficiency of miR-582-3p inhibitor was detected by qRT-PCR. After K562 and HL-60 cells were introduced with si-NC, si-circ-SFMBT2, si-circ-SFMBT2+anti-miR-NC or si-circ-SFMBT2+anti-miR-582-3p, miR-582-3p level (B), cell viability (C), the number of colonies (D), apoptosis rate (E), cell migration and invasion (F and G) were examined by qRT-PCR, CCK-8, colony formation, flow cytometry and transwell assays. (H and I) After transfection, the levels of CyclinD1, Bax and MMP9 were determined by Western blot. (J-L) Relative glucose consumption, lactate production and ATP/ADP ratios were tested using the corresponding kit. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 5. MiR-582-3p directly targeted ZBTB20. (A) The predicted binding site of miR-582-3p in ZBTB20 3’UTR was exhibited by TargetScan. (B and C) Dual-luciferase reporter assay was used to validate the binding relationship. (D) ZBTB20 mRNA level in serum from AML patients (n=27) and healthy volunteers (n=27) was detected by qRT-PCR. (E) TCGA data showed that ZBTB20 was up-regulated in AML.
patients. (F) Spearman’s correlation analysis was utilized to detect the correlation between miR-582-3p and ZBTB20. (G) ZBTB20 protein level was measured by Western blot in HS-5 cells and AML cells (K562 and HL-60). (H) ZBTB20 protein level was examined by Western blot in K562 and HL-60 cells transfected with miR-NC, miR-582-3p, anti-miR-NC or anti-miR-582-3p. **P < 0.01, ***P < 0.001, ****P < 0.0001.

**Figure 6. Silencing of ZBTB20 suppressed the malignant phenotypes of AML cells.** K562 and HL-60 cells were introduced with si-NC or si-ZBTB20, respectively. ZBTB20 protein level (A), cell viability (B), the number of colonies (C), cell apoptosis (D), migration and invasion (E and F) were evaluated by Western blot, CCK-8, colony formation, flow cytometry and transwell assays. (G and H) The levels of CyclinD1, Bax and MMP9 were measured using Western blot after transfection. (I-K) Glycolysis was assessed by detecting glucose consumption, lactate production and ATP/ADP ratios. ***P < 0.001, ****P < 0.0001.

**Figure 7. ZBTB20 abolished the effect of miR-582-3p on AML cell progression.** (A) The overexpression efficiency of ZBTB20 was confirmed by Western blot. K562 and HL-60 cells were divided into four groups: miR-NC, miR-582-3p, miR-582-3p+pcDNA and miR-582-3p+ZBTB20. ZBTB20 protein expression (B), cell viability (C), the number of colonies (D), apoptosis rate (E), cell migration and invasion (F and G) and the levels of related proteins (H and I) were examined using appropriate methods. (J-L) Relative glucose consumption, lactate production and ATP/ADP ratios were detected via the corresponding kit. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

**Figure 8. Circ-SFMBT2 regulated ZBTB20 expression by sponging miR-582-3p.** (A and B) K562 and HL-60 cells were transfected with si-NC, si-circ-SFMBT2, si-circ-
SFMBT2+anti-miR-NC or si-circ-SFMBT2+anti-miR-582-3p, and ZBTB20 protein level was measured by Western blot. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. 
A

WT-circ-SFMBT2 5' GAUACAGACUUCG ACCAGUUG 3'
miR-582-3p 3' CCAAGUCAAAAGU UGGUCAA U 5'
MUT-circ-SFMBT2 5' GAUACAGACUUCG UGGUCAA G 3'

B

C

D

E

F

G

H

I

r=-0.6064
p<0.001
A. Relative miR-582-3p expression in K562 and HL-60 cells. B. Relative miR-582-3p expression in K562 and HL-60 cells. C. Cell viability (%) in K562 and HL-60 cells. D. Number of colonies in K562 and HL-60 cells. E. Apoptosis rate (%) in K562 and HL-60 cells. F. Number of migrated cells in K562 and HL-60 cells. G. Number of invasive cells in K562 and HL-60 cells. H. Relative protein expression of CyclinD1, Bax, MMP9, and GAPDH in K562 and HL-60 cells. I. Relative protein expression of CyclinD1, Bax, and MMP9 in HL-60 cells. J. Relative glucose consumption in K562 and HL-60 cells. K. Relative lactate production in K562 and HL-60 cells. L. Relative ATP/ADP ratios in K562 and HL-60 cells.
**HISTOLOGY AND HISTOPATHOLOGY**

A. Schematic diagram showing the interaction between miR-582-3p and ZBTB20 3'UTR. **B.** Relative luciferase activity of K562 cells transfected with WT-ZBTB20 3'UTR and MUT-ZBTB20 3'UTR with miR-NC and miR-582-3p. **C.** Relative luciferase activity of HL-60 cells transfected with WT-ZBTB20 3'UTR and MUT-ZBTB20 3'UTR with miR-NC and miR-582-3p. **D.** Box plot showing the relative ZBTB20 mRNA expression in normal and AML samples. **E.** Relative ZBTB20 mRNA expression in LAMCL and AML samples. **F.** Scatter plot showing the correlation between relative miR-582-3p expression and ZBTB20 mRNA expression. **G.** Western blot analysis showing ZBTB20 protein expression in HS-5, K562, and HL-60 cells. **H.** Western blot analysis showing ZBTB20 protein expression in K562 and HL-60 cells transfected with miR-NC, miR-582-3p, anti-miR-NC, and anti-miR-582-3p.
**HISTOLOGY AND HISTOPATHOLOGY**

**A**

![Bar graphs showing relative ZBTB20 protein expression in K562 and HL-60 cells treated with si-NC or si-ZBTB20.](image)

**B**

![Bar graphs showing cell viability in K562 and HL-60 cells treated with si-NC or si-ZBTB20.](image)

**C**

![Bar graphs showing number of colonies in K562 and HL-60 cells treated with si-NC or si-ZBTB20.](image)

**D**

![Bar graphs showing apoptosis rate in K562 and HL-60 cells treated with si-NC or si-ZBTB20.](image)

**E**

![Bar graphs showing number of migrated cells in K562 and HL-60 cells treated with si-NC or si-ZBTB20.](image)

**F**

![Bar graphs showing number of invasive cells in K562 and HL-60 cells treated with si-NC or si-ZBTB20.](image)

**G**

![Western blots showing expression of CyclinD1, Bax, MMP9, and GAPDH in K562 and HL-60 cells treated with si-NC or si-ZBTB20.](image)

**H**

![Western blots showing relative protein expression of CyclinD1, Bax, MMP9, and GAPDH in K562 and HL-60 cells treated with si-NC or si-ZBTB20.](image)

**I**

![Bar graphs showing relative glucose consumption in K562 and HL-60 cells treated with si-NC or si-ZBTB20.](image)

**J**

![Bar graphs showing relative lactate production in K562 and HL-60 cells treated with si-NC or si-ZBTB20.](image)

**K**

![Bar graphs showing relative ATP/ADP ratios in K562 and HL-60 cells treated with si-NC or si-ZBTB20.](image)